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Modification of olivomycin A at the side chain of the aglycon yields the derivative with perspective antitumor characteristics

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ABSTRACT

A novel way of chemical modification of the antibiotic olivomycin A (**1**) at the side chain of the aglycon moiety was developed. Interaction of olivomycin A with the sodium periodate produced the key acid derivative olivomycin SA (**2**) in 86% yield. This acid was used in the reactions with different amines in the presence of benzotriazol-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate (PyBOP) or diphenylphosphoryl azide (DPPA) to give corresponding amides. Whereas olivomycin SA was two orders of magnitude less cytotoxic than the parent antibiotic, the amides of **2** demonstrated a higher cytotoxicity. In particular, *N,N*-dimethylaminoethylamide of olivomycin SA showed a pronounced antitumor effect against transplanted experimental lymphoma and melanoma and a remarkably high binding constant to double stranded DNA. The therapeutic effects of this derivative were achievable at tolerable concentrations, suggesting that modifications of the aglycon's side chain, namely, its shortening to methoxyacetic residue and blocking of free carboxyl group, are straightforward for the design of therapeutically applicable derivatives of olivomycin A.

1. Introduction

Olivomycin A (olivomycin I) belongs to the family of aureolic acid antibiotics that includes also mithramycin, chromomycin A3 and durhamycin.[1] These remarkably cytotoxic compounds bind to GC-rich regions in the DNA minor groove, thereby interfering with vital cellular functions. Thus, the aureolic acid derivatives emerge as a promising chemical class in antitumor drug design.[1–3] Attempts to design the practically applicable aureolic acid-based agents have been aimed at widening a 'therapeutic window', that is, an attenuation of general toxicity in vivo and retention of the cytotoxic potency. Earlier we have described the chemical modification of olivomycin A at the position 5 of the aglycon moiety accompanied by splitting off the disaccharide branch in the position 6. The resulting compounds were less cytotoxic than the parent antibiotic.[4] In contrast, modification of olivomycin A at the 2′-keto group of the aglycon's side chain produced active compounds. One derivative, 2-adamantylamide of 2′-(carboxymethoxime)olivomycin A, showed reasonable potency and a lower general toxicity than olivomycin A in mice bearing P388 lymphoma transplants.[5] These results suggested that the modifications focused on the aglycon's side chain of olivomycin A can yield the derivatives with improved antitumor characteristics.

Furthermore, the biosynthetic analogs of mithramycin and chromomycin A3 with shorter, functionally different side chains of the aglycon have been obtained by combinatorial biosynthetic procedures in the producing organisms.[6–8] Chromomycin A3 and mithramycin derivatives with various substituents in the side chain attached to C-3 of the aglycon (Fig. 1) have been obtained by genetic engineering methods and separated by HPLC. The compounds with the shortest side chain (mithramycin SA; MSA or chromomycin SA; CSA) have been isolated in low yields. Mithramycin SDK and chromomycin SDK showed the highest affinity to DNA. It has been suggested that a lower affinity to DNA of MSA and CSA was due to the presence of carboxyl groups at the end of the C-3 side chain.[7] The negative charge of these moieties at neutral pH may hamper the interaction of the entire molecule with DNA. These results clearly showed that the aliphatic side chain of the aglycon of olivomycin A is a promising target for the preparation of novel semisynthetic congeners with potential therapeutic applications.

In the present study we synthesized olivomycin SA, an analog of olivomycin A similar to MSA and CSA, and transformed it into the amide derivatives devoid of negatively charged carboxyl groups.

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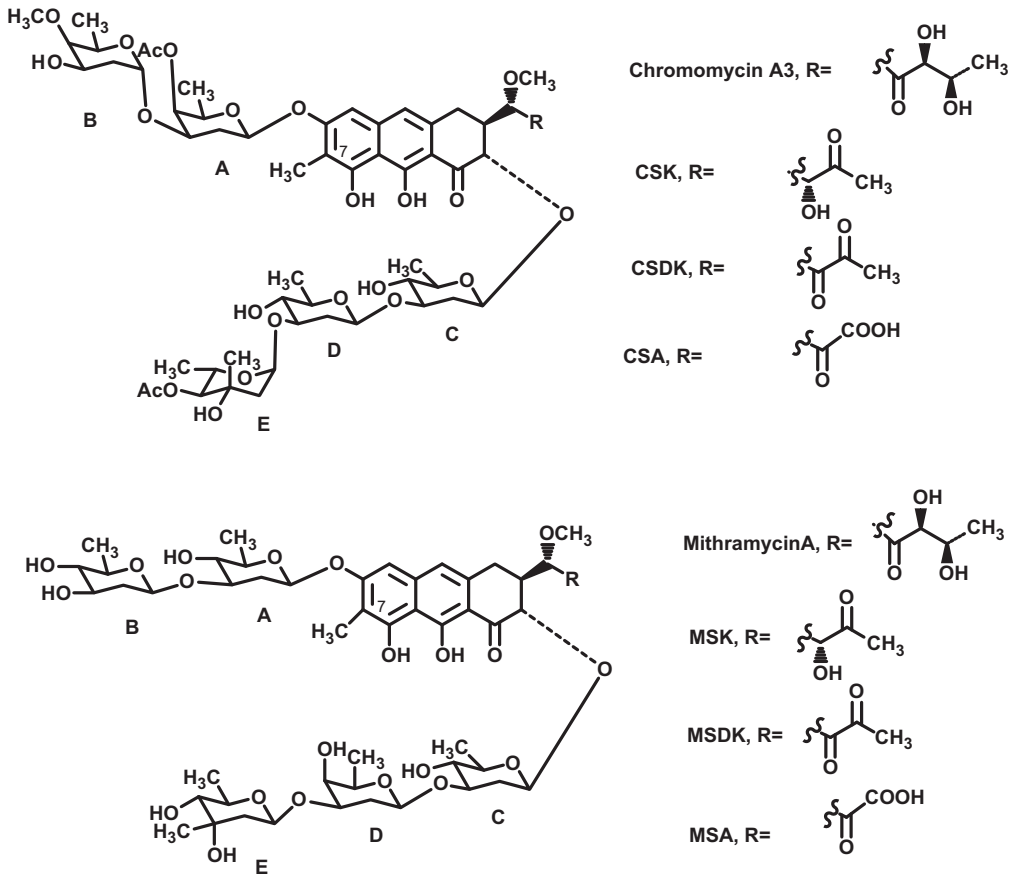


Figure 1. Structures of chromomycin A3 and mithramycin derivatives obtained by combinatorial biosynthesis. MTA, mithramycin A; MSK, mithramycin SK; MSDK, mithramycin SDK; MSA, mithramycin SA; CRO, chromomycin A3; CSK, chromomycin SK; CSDK, chromomycin SDK; CSA, chromomycin SA.

Importantly, *N,N*-dimethylaminoethylamide of olivomycin SA demonstrated good antiproliferative potency against cultured human tumor cell lines as well as therapeutic efficacy in vivo against transplanted murine tumors. These activities were paralleled by a high affinity of the novel derivative to double stranded DNA.

2. Results

2.1. Synthesis

We developed a novel method of chemical modification of olivomycin A (**1**) based on the oxidation of vicinal hydroxyl and keto groups in the aglycon moiety with sodium periodate. The key derivative 3-*C*-(3*S*)-6-{[4-*O*-acetyl-2,6-dideoxy-3-*O*-(2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranosyl)-β-D-*lyxo*-hexopyranosyl]oxy}-2-{[4-*O*-isobutyroyl-2,6-dideoxy-3-*C*-methyl-α-L-*arabino*-hexopyranosyl-(1→3)-β-D-*arabino*-hexopyranosyl-(1→3)-β-D-*arabino*-hexopyranosyl]oxy}-8,9-dihydroxy-(1-oxo-1,2,3,4-tetra-hydroanthracen-2-yl)-2-*O*-methyl-acetic acid (compound **2**) possesses (*S*)-(carboxy)(methoxy)methyl substituent in the position C3. The sugar substituents and the aglycon (olivin) nucleus were unaltered in this reaction. By analogy with MSA or CSA, compound **2** can be called olivomycin SA. The ^{1}H and ^{13}C NMR spectra of **2** identified all signals of hydrogen and carbon atoms in the aglycon and carbohydrate moieties (Table 1). The assignment of signals in NMR spectra of **2** was performed using ^{1}H–^{1}H homonucleus double resonance method and the literature data for chromomycin SA.[8] The acid **2** further reacted with various amines in the presence of benzotriazol-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate (PyBOP) or diphenylphosphoryl azide (DPPA) to give the corresponding amides **3**–**10** (Scheme 1). Thin-layer chromatography (TLC) and HPLC analyses showed that compounds **2**–**10** were homogeneous. The structures of all new derivatives were confirmed by IR spectroscopy and high-resolution mass spectrometry (HR-MS) (Table 2). Because compound **3** contained the tertiary amino group, we obtained L-glutamate of **3** (compound **3a**) that was readily soluble in water. Compounds **3** and **3a** were identical by HPLC (see Supplementary data). The structure of **3a** was confirmed by elemental analysis.

2.2. Characterization of antitumor properties

2.2.1. Antiproliferative potency against human tumor cell lines

The antiproliferative potency of novel compounds and the parent antibiotic **1** was tested in human HCT116 (colon carcinoma) and K562 (leukemia) cell lines after a 72 h exposure of cells to the compounds followed by MTT-test.[9] As shown in Table 3, compound **2** was two orders of magnitude less potent than the parent antibiotic **1**. Amidation of **2** led to a substantially increased potency; moreover, **3**, **9** and **1** were similarly potent, their cytotoxicity was achievable at nanomolar concentrations. The water-soluble salt **3a** was as active as **3**. In contrast, D-galactosamine derivative **10** turned out to be virtually non-toxic. For further studies we selected compound **3a**.

2.2.2. Drug–DNA complex formation

Given that binding to DNA is a critical parameter of cytotoxicity of aureolic acid derivatives,[10,11] we studied the formation of complexes of **3a** with double stranded DNA. Figure 2A and B show the fluorescence spectra of drug–DNA complexes in the buffer with

Table 1
The ^{1}H and ^{13}C NMR spectra for compound **2** ($CDCl_3$–CD_3OD, 10:1, 20 °C)

Atom	^{13}C (ppm)	^{1}H (ppm) (*J*, Hz)
Aglycon		
C1	203.0	—
C2	76.5	4.69, d (11.8)
C3	43.9	2.65, m, overlap
C4	27.5	3.13, d (16.7); 2.76, dd (16.7, 3.8)
C4a	136.5	—
C5	101.96	6.57, br d (2.2)
C6	160.6	-
C7	102.6	6.53, d (2.2)
C8	157.2	-
C8a	108.2	-
C9	164.5	—
C9a	108.2	—
C10	117.2	6.75, br s
C10a	140.8	—
C1′	76.6	4.33, d (1.8)
C2′	174.9	—
C1′-OCH_3	59.2	3.53, s
Sugar A		
A1	96.8	5.24, dd (9.6, 2.5)
A2	32.55[a]	2.12, dd (10.5, 2.5); 2.08, dd (10.0, 4.5)
A3	69.8	4.0, m overlap
A4	67.2	5.16, d (2.9)
A5	69.4	3.83, q (6.6)
CH_3CO	20.5	2.18, s
CH_3CO	171.4	—
A6	16.4	1.29, d (6.5)
Sugar B		
B1	95.0	5.12, d (3.1)
B2	32.62[a]	1.83, dt (12.4, 3.5); 1.71, dd (12.9, 4.5)
B3	65.6	3.98, ddd (12.1, 5.2, 2.8)
B4	81.2	3.24, d (2.8)
B5	66.8	3.89, q (6.5)
B6	16.8	1.28, d (6.4)
B4-OCH_3	61.8	3.60, s
Sugar C		
C1	100.2	5.05, dd (9.3, 2.0)
C2	37.0	2.51, dd (12.8, 4.9); 1.67, m
C3	81.6	3.68[b]
C4	77.8	3.12, dd (8.7, 8.7)
C5	71.8	3.31, dq (9.2, 6.1)
C6	17.7	1.37, d (6.0)
Sugar D		
D1	99.2	4.64, dd (9.6, 1.8)
D2	36.6	2.31, dd (12.7, 5.0); 1.66, m
D3	78.9	3.56, under H_2O
D4	74.7	3.13, dd (8.8, 8.8)
D5	72.2	3.41, dq (3.1, 6.1)
D6	17.6	1.39, d (6.0)
Sugar E		
E1	96.1	5.03, dd (4.3, 1.8)
E2	43.3	2.06, m; 2.01, dd (10.5, 4.0)
E3	70.0	—
E4	78.5	4.63, d (9.2)
E5	66.6	4.06, dq (9.1, 6.1)
E6	17.4	1.22, d (6.2)
E3-CH_3	22.4	1.37, s
$(CH_3)_2CHCO$	37.0	2.64, m (7.0)
$(CH_3)_2CHCO$	18.61, 18.58	1.21, d (7.0)
$(CH_3)_2CHCO$	177.6	—

[a] Reverse assignment of signals is possible.
[b] Under residual OH from CD_3OD.

physiological values of pH, ionic strength and temperature. The addition of novel compounds to DNA solution followed by excitation at 420nm resulted in a dose-dependent increase of fluorescence at $\lambda = 530$ nm, indicating the formation of drug–DNA complexes (Fig. 2A and B). Importantly, the Scatchard plots (Fig. 2C) demonstrated that the binding constant of **3a** was substantially bigger than the respective parameter for its precursor **2** (1.35×10^5 vs 2.1×10^4 M^{-1}, respectively).

2.2.3. Antitumor efficacy in animal models

We studied the antitumor efficacy of the water-soluble derivative **3a** in mice bearing P388 lymphoma transplants. The parent antibiotic **1** was used as a reference agent. As shown in Table 4, injections of **1** (2 mg/kg daily for 5 days) resulted in 35% increase of lifespan (ILS); dose escalation did not prolong the lifespan but caused a high (87% of mice) lethality due to drug toxicity. Treatment with **3a** (4 mg/kg daily for 5 days) resulted in a longer survival of tumor-bearing animals (ILS = 52%); no drug-related deaths were registered with this regimen. The therapeutic effect of **3a** included the retardation of tumor ascite growth determined as a slower increase of body weight (data not shown).

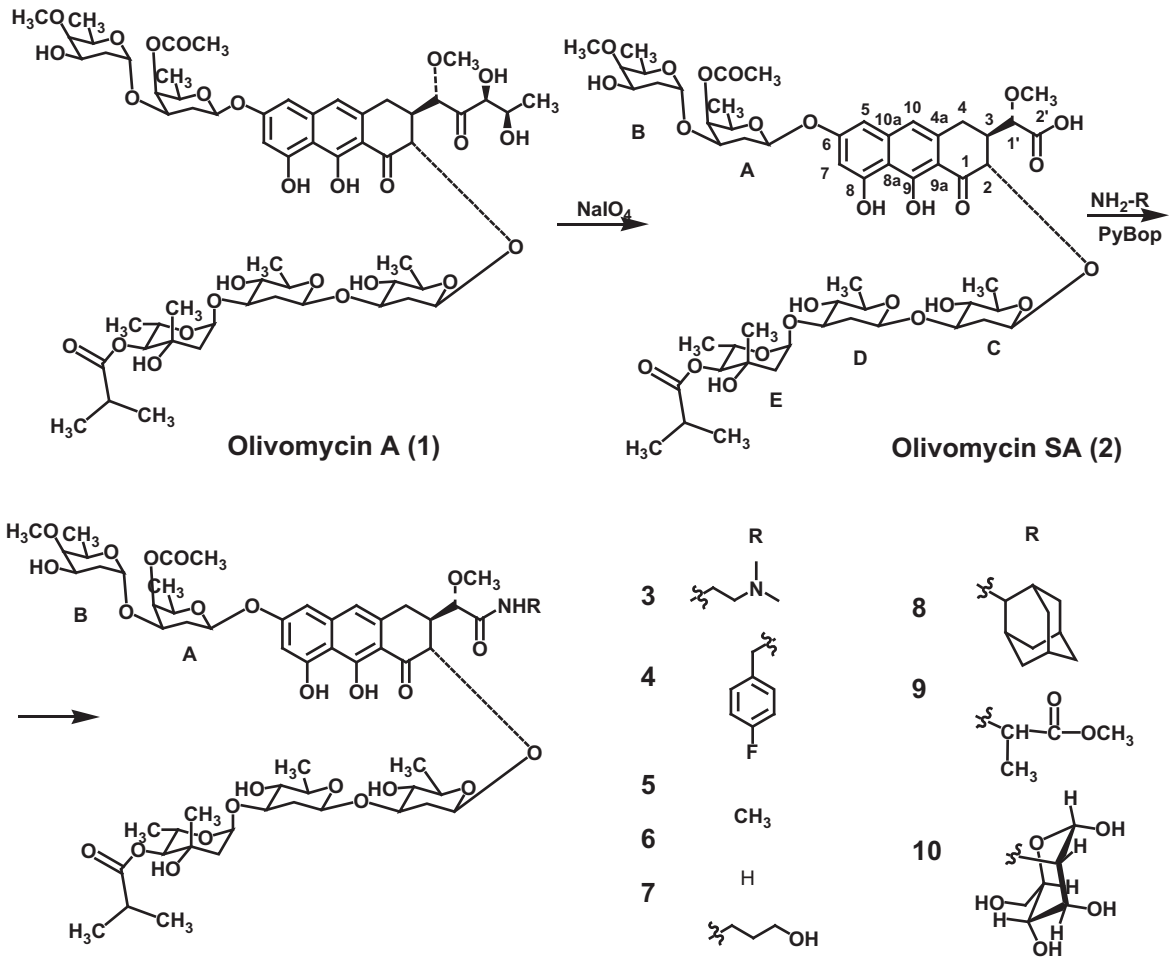
Next, **3a** and **1** were tested in mice bearing B16 melanoma transplants. Compound **3a** was therapeutically inactive at 2 and 4 mg/kg but demonstrated an antitumor potency at 6 and 8 mg/kg: values of inhibition of tumor growth (ITG) were 64% and 47%, respectively. The best results were achieved with 10 mg/kg of **3a** (both 5 and 8 injection regimens): ITG = 73% and 85%, respectively (Table 5). No drug related deaths were registered in mice treated with **3a**.

Figure 3 shows the time course of tumor growth in B6D2F1 mice bearing B16 melanoma transplants that received 8 injections of **1** (optimal therapeutic dose 2 mg/kg per injection) or **3a** (optimal therapeutic dose 10 mg/kg per injection; Table 4). Compound **3a** demonstrated a substantial advantage over **1**: the mean calculated tumor weight (CTW) was significantly smaller in mice treated with **3a** at days 21 ($p = 0.055$), 25 ($p = 0.02$) and 28 ($p = 0.03$).

3. Discussion

In an effort to obtain new analogs of olivomycin A (**1**) with improved antitumor properties, we developed a novel method of chemical modification of **1** at the side chain of the antibiotic's aglycon moiety. The key intermediate olivomycin SA (**2**) contains the carboxyl group suitable for further modifications that resulted in a series of amides of **2**. The structure of the amine residue influenced the antiproliferative activity of new derivatives. While **2** was two orders of magnitude less cytotoxic than **1** against HCT116 and K562 cell lines, amidation of **2** yielded the compounds some of which were as potent as **1**. Remarkably, **3a** demonstrated a high antitumor efficacy in two models of aggressive tumors such as P388 lymphoma and B16 melanoma. The increased lifespan and the inhibition of tumor growth by **3a** were associated with no signs of general toxicity such as drug-related death, hair and weight loss, or changes in behavior of mice. These results strongly suggested that the modification of the side chain of the aglycon in **1** is straightforward for the development of aureolic acid derivatives as tentative antitumor agents.

The double stranded DNA is a major intracellular target critical for antiproliferative effect of aureolic acid-based compounds. Differential affinity of **2** and **3a** to DNA correlated with their antiproliferative potencies against studied tumor cell lines. It is plausible to hypothesize that transition from free acid **2** to an amide and especially to the amides with an additional dimethylamino group (**3** and **3a**) facilitates the positioning of the compound in the DNA minor groove. Indeed, the presence of dimethylamino group led to ~6.4-fold increase of the DNA binding constant of **3a** compared to **2**. Recently we have demonstrated that the derivative of 2′-(carboxymethoxime)olivomycin A with a bulky adamantylamide substituent was less toxic than **1**; accordingly, the binding constant of the latter derivative to DNA was lower than that of **1**.[5,12] A detailed analysis of quantitative parameters and drug-DNA interaction modes of **1** and its derivatives with various substituents in the aglycon's side chain is required to identify the SAR factors crucial for design of clinically applicable compounds.



Scheme 1. Chemical transformations of olivomycin A and synthesis of olivomycin SA amides.

Table 2
The HPLC, IR and mass spectrometry data for compounds **1**–**10**

Compound	Rt (min)	IR (nm)	HR ESI-MS	
			Calculated MW	Found
1	25.0	1738, 1640, 1582, 1510, 1056	$C_{58}H_{84}O_{26}$ 1196.5250	$[M+Na]^{+1}$ 1219.5150
2	25.7	1738, 1637, 1582, 1515, 1445, 1064	$C_{55}H_{78}O_{25}$ 1138.4832	$[M-H]^{-1}$ 1137.3487
3	16.5	1737, 1633, 1582, 1531, 1454, 1061	$C_{59}H_{88}N_2O_{24}$ 1208.5727	$[M+H]^{+1}$ 1209.5801
4	29.9	1738, 1636, 1582, 1511,1491, 1061	$C_{62}H_{84}FNO_{24}$ 1245.5367	$[M+Na]^{+1}$ 1268.5309
5	25.0	1738, 1660, 1633, 1579, 1532, 1061	$C_{56}H_{81}NO_{24}$ 1151.5148	$[M+Na]^{+1}$ 1174.5453
6	24.2	1736,1673, 1633,1584,1536, 1450, 1063	$C_{55}H_{79}NO_{24}$ 1137.4992	$[M+Na]^{+1}$ 1160.3487
7	23.7	1737, 1633, 1536, 1449, 1063	$C_{58}H_{85}NO_{25}$ 1195.5410	$[M+Na]^{+1}$ 1218.5711
8	38.3	1738, 1619, 1525, 1452, 1062	$C_{65}H_{93}NO_{24}$ 1271.6087	$[M+Na]^{+1}$ 1294.6401
9	24.7	1741, 1635, 1582, 1518, 1455, 1062	$C_{59}H_{85}NO_{26}$ 1223.5359	$[M+Na]^{+1}$ 1246.5559
10	20.7	1732, 1634, 1582, 1521, 1456, 1062	$C_{61}H_{89}NO_{29}$ 1299.5520	$[M-H]^{-1}$ 1298.5517

4. Conclusion

Our data provide evidence that the newly developed modification of the side chain of the aglycon in **1** is promising for obtaining practically suitable agents in this chemical class. Regardless of their marked apoptotic potency for a variety of tumor cells and the ability to interfere with gene expression mediated by GC-binding transcription factors,[13] the aureolic acid family antibiotics remain largely experimental tools.[14–16] Importantly, MSDK and MSK have been shown to be well tolerated after prolonged administration; these compounds delayed growth of ovarian tumor xenografts, leading to a significant increase of survival.[17] Our study demonstrates that the modifications of the side chain, in particular, its shortening to methoxyacetic residue and blocking of free carboxyl group, can yield a derivative of **1** with superior DNA binding constant and good therapeutic characteristics.

5. Experimental

5.1. Chemistry

5.1.1. General experimental procedures

Olivomycin A (**1**) was produced at the pilot plant of Gause Institute of New Antibiotics, Moscow. Sodium periodate, benzotriazol-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate (PyBOP) and diphenylphosphoryl azide (DPPA) were

Table 3
The antiproliferative potency of olivomycin A (**1**) and derivatives **2**–**10** for HCT116 and K562 cell lines

Compound/cell line	IC_{50}[a] (nmol)	
	HCT116	K562
1	20 ± 2[a]	50 ± 4
2	2000 ± 138	2200 ± 165
3	20 ± 3	47 ± 5
3a	40 ± 5	53 ± 6
4	278 ± 45	148 ± 18
5	120 ± 14	ND[b]
6	100 ± 8	ND
7	156 ± 15	ND
8	180 ± 21	ND
9	20 ± 4	63 ± 7
10	>12,500	2388 ± 340

[a] IC_{50}–concentration that caused 50% growth inhibition after 72 h of exposure.
[b] ND–not tested, data are mean ± SD of 4–5 independent experiments.

Table 4
The antitumor efficacy of **1** and **3a** in P388 lymphoma bearing B6D2F1 mice

Treatment	Daily dose (mg/kg)	ILS (%)
Olivomycin A (**1**)	4	[a]
	2	35
	1	24
Compound **3a**	4	52
	2	28
	1	14

Animals were treated with the indicated doses of **1** or **3a** administered ip daily for 5 days. The increase of lifespan (ILS) was calculated as described in Section 5.
[a] In this group 87% of mice died of drug-related toxicity.

purchased from Acros (Belgium). Other reagents and solvents were from Sigma–Aldrich (USA) or Merck (Germany) unless specified otherwise. Reaction products were purified by column chromatography on Merck silica gel G60 (0.040–0.063 μm). The progress of reactions, column eluates and all final samples were analyzed by TLC and HPLC. TLC was performed on Merck $G60F_{254}$ precoated plates in the following systems: $CHCl_3$–MeOH–CH_3COOH, 10:1:0.01 (system A) and $CHCl_3$–MeOH–CH_3COOH, 7:1:0.01 (system B). The spots were detected by sight and with a UV lamp at 254 nm. Melting points were determined on Buchi SMP-20 and are uncorrected. Analytical HPLC was performed on a Shimadzu HPLC instrument the SPD-M20A series (Japan) on a Gemini-110A-C18 column (4.6 × 250 mm, particle size 5 μm, Phenomenex, USA) at an injection volume 20 μl (concentration of samples was 0.02–0.03 mg/ml) and at a wavelength 270 nm. The system contained 0.01 N H_3PO_4 (pH 2.6) with acetonitrile. The proportion of acetonitrile varied from 20% to 80% for 30 min, and from 80% to 90% for 10 min with a flow rate of 1.0 ml/min at 20 °C. The IR spectra were obtained on a Nicolet-iS10 Fourier transform IR spectrometer (DTGS detector, splitter KBr) with a Smart Performer module

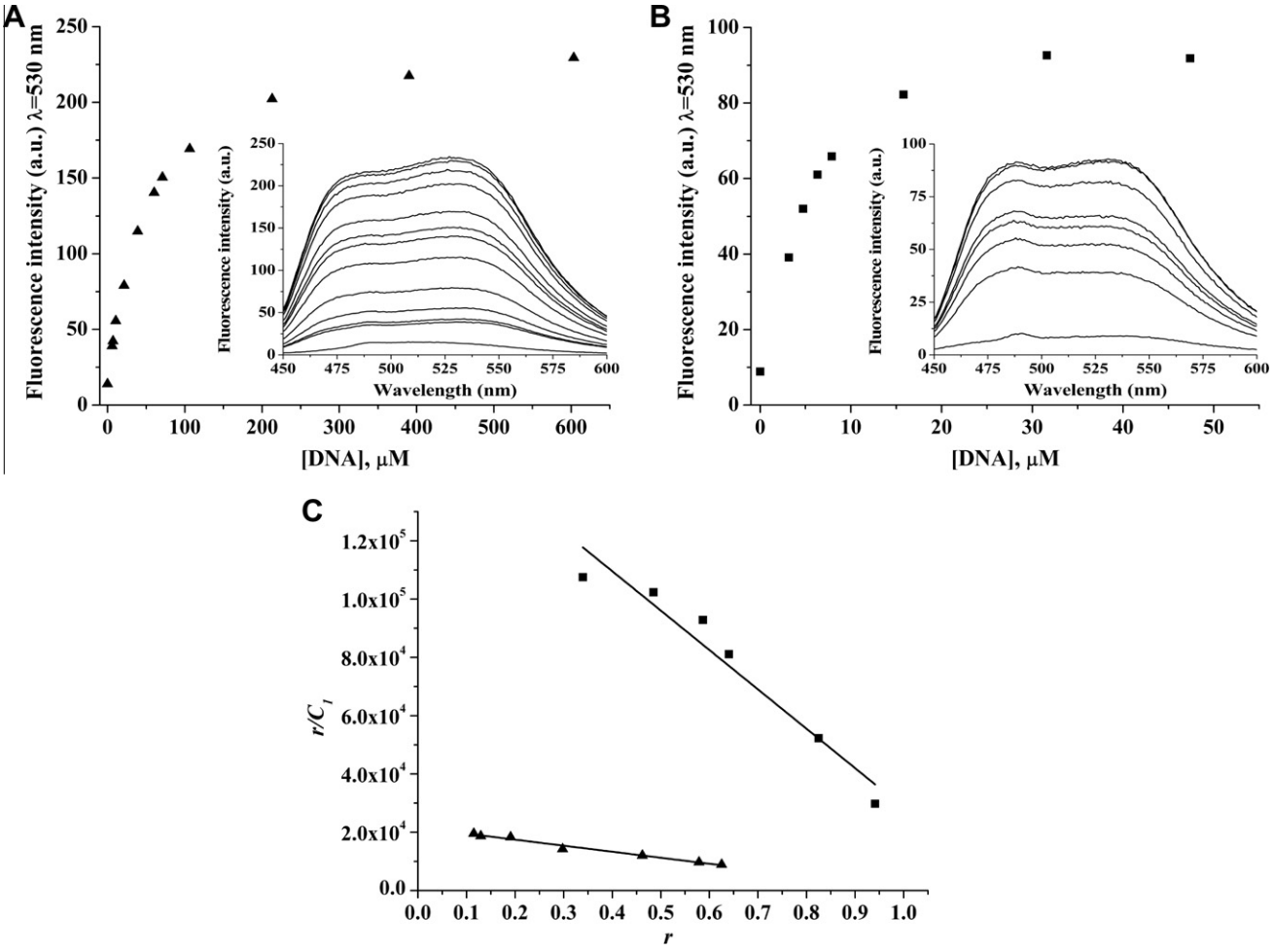


Figure 2. Binding isotherms of compounds **2** and **3a** at different DNA concentrations. (A, B) maximal fluorescence intensities at λ = 530 nm for **2** (A) and **3a** (B) in the buffer containing 10 mM Tris–HCl, pH 7.5 and 50 mM $MgCl_2$. Insets, fluorescence titration curves. (C), Scatchard plots for **2** (triangles) and **3a** (squares). The solid lines show the best fitting curves. See Section 5 for details.

Table 5
The antitumor efficacy of **1** and **3a** in B6D2F1 mice bearing B16 melanoma transplants

Treatment	Dose (mg/kg×times)	ITG (%)
Olivomycin A (**1**)	2 × 5	48
	2 × 8	45
Compound **3a**	10 × 8	85
	10 × 5	73
	5 × 8	52

Mice were treated with the indicated doses of **1** or **3a** administered iv (5 or 8 day regimens). The inhibition of tumor growth (ITG) was calculated as described in Section 5.

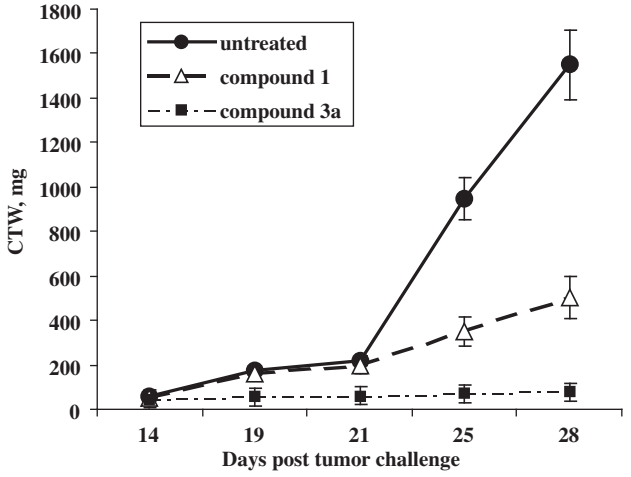


Figure 3. Time course of tumor growth in B16 melanoma bearing B6D2F1 mice treated with **1** or **3a**. Animals received 8 injections of **1** or **3a** at the most efficacious (optimal) doses: 2 and 10 mg/kg, respectively. The calculated tumor weight (CTW) was determined as described in Section 5. See the text for statistical differences between the experimental groups.

equipped with a ZnSe crystal. The spectra were run at the range 3000–650 cm^{-1} with resolution 4 cm^{-1}. The spectra were analyzed using OMNIC-7.0 program package.

The ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR-400 NMR spectrometer at 20 °C and referenced to TMS observing protons at 400 MHz and ^{13}C at 100 MHz. High resolution electrospray mass spectra were recorded on a Bruker micrOTOF-Q II instrument (Bruker Daltonics GmbH, Germany). Data on predominant monoisotope peaks are presented. All solutions were dried over sodium sulfate and evaporated at reduced pressure on a Buchi rotary evaporator at the temperature below 35 °C.

5.1.2. Olivomycin SA (2)

A solution of sodium periodate (200 mg, 0.935 mmol) in H_2O (0.5 ml) was added to a solution of **1** (500 mg, 0.418 mmol) in MeOH (5 ml). The reaction mixture was stirred at room temperature for 24 h and then diluted with ethyl acetate (50 ml). The ethyl acetate fraction was washed with H_2O (3 × 30 ml), dried over Na_2SO_4 and evaporated. The crude residue was purified by column chromatography on silica gel and eluted with $CHCl_3$–MeOH–CH_3COOH (15:1:0.1). The resulting fractions were combined and evaporated to a small volume. The addition of hexane gave a precipitate that was filtered off, washed with hexane and dried in vacuum to yield **2** as amorphous powder (410 mg, 86%). R_f 0.58 (system A), mp 170–172 °C (decomp.).

5.1.3. *N,N*-Dimethylaminoethylamide of olivomycin SA (3)

N,N-Dimethylaminoethylamine hydrochloride (49 mg, 0.395 mmol) was added to the solution of **2** (150 mg, 0.131 mmol) in DMSO (3 ml). Et_3N was added to the reaction mixture to adjust pH to 7.5–8.0. Then PyBOP (103 mg, 0.263 mmol) was added, the reaction mixture was stirred at room temperature for 40 min. Ether (20 ml) was added, the reaction mixture was stirred vigorously, and the upper layer was removed. Ethyl acetate (20 ml) was added to the obtained viscous yellow oil. The white precipitate was filtered off; the ethyl acetate solution was concentrated in vacuum to ~1 ml volume. The addition of petroleum ether gave a precipitate that was filtered off, washed with petroleum ether and dried in vacuum to yield **3** as amorphous powder (130 mg, 82%). R_f 0.33 (system B), mp 175–177 °C (decomp.).

5.1.4. 4-Fluorobenzylamide of olivomycin SA (4)

4-Fluorobenzylamine hydrochloride (8 mg, 0.03 mmol) was added to the solution of **2** (10 mg, 0.01 mmol) in DMF (0.5 ml). Et_3N was added to the reaction mixture to adjust pH to 7.5–8.0, and then DPPA (0.012 ml, 0.06 mmol) was added. The reaction mixture was stirred at room temperature for 2 h. Ether (2 ml) was added, the mixture was stirred vigorously; then the upper ethereal layer was removed. The addition of petroleum ether to the obtained viscous yellow oil gave a precipitate that was centrifuged and dried to yield target **4** as amorphous powder (7 mg, 70%). R_f 0.33 (system B), mp 165–167 °C (decomp.).

5.1.5. Other amides of olivomycin SA (5–10)

Methylamide of olivomycin SA (**5**), amide of olivomycin SA (**6**), 3-hydroxypropylamide of olivomycin SA (**7**), adamantylamide of olivomycin SA (**8**), L-alanylamide methylate of olivomycin SA (**9**) and D-galactosamide of olivomycin SA (**10**) were obtained starting from **2** and the corresponding amine hydrochloride in DMSO or DMF in the presence of PyBOP and Et_3N. The purity of **5**–**10** was determined by TLC and HPLC. The structures of **2**–**10** were confirmed by IR spectroscopy and HR-ESI mass-spectrometry (Table 2).

5.1.6. L-Glutamate of *N,N*-dimethylaminoethylamide of olivomycin SA (3a)

A solution of L-glutamic acid (12 mg, 0.083 mmol) in H_2O (0.5 ml) was added to a solution of *N,N*-dimethylaminoethylamide of olivomycin SA (**3**) (100 mg, 0.083 mmol) in MeOH (3 ml). The mixture was stirred for 15 min at room temperature, and then acetone (10 ml) was added. The mixture was concentrated to a small volume (~0.5 ml). Then acetone (1 ml), ethyl acetate (1 ml) and petroleum ether (10 ml) were added. The precipitate was filtered off, washed with petroleum ether and dried. Yield 100 mg (90%). HPLC data in an acidic buffer for compounds **3** and **3a** were identical (see Supplementary data). Elemental analysis: calculated for $C_{67}H_{97}N_3O_{28}$ C 57.79, H 7.02, N 3.02, O 32.17, found C 57.55, H 7.12, N 2.98.

5.2. Biology

5.2.1. Cell lines and viability assays

The HCT116 human colon carcinoma and K562 human leukemia cell lines (American Type Culture Collection; ATCC) were cultured in Dulbecco's modified Eagle medium or RPMI-1640 supplemented with 5% fetal calf serum (HyClone, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO_2 in humidified atmosphere. Cells in logarithmic phase of growth were used in all experiments. The tested compounds were dissolved in DMSO as 10 mM stock solutions followed by serial dilutions in water immediately before experiments. The antiproliferative potencies were determined in a formazan conversion assay (MTT-test).[9] Cells (5×10^3 in 190 μl of culture medium) were plated into a 96-well plate (Becton Dickinson, USA) and treated with 0.1% DMSO (vehicle control) or with increasing concentrations of tested compounds (each dose in duplicate) for 72 h. After the completion

of drug exposure, 50 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added into each well for an additional 2 h. Formazan was dissolved in DMSO, and the absorbance at λ = 540 nm was registered. Cell viability at a given drug concentration was calculated as the percentage of absorbance in wells with drug-treated cells to that of vehicle control cells (100%). The IC_{50} (a 50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell viability by 50%.

5.2.2. Drug–DNA complex formation

Compounds **2** (1.34 μM) and **3a** (0.8 μM) were added to the solution of salmon sperm double stranded DNA (Technomedservice, Moscow) in the buffer containing 10 mM Tris–HCl, pH 7.5 and 50 mM $MgCl_2$. The concentration of DNA pairs was determined using the molar extinction coefficient (6.7×10^3 M^{-1} cm^{-1}). Fluorescence spectra of the complexes of **2** and **3a** with DNA ($450 < \lambda < 600$ nm) were registered on a Shimadzu RF-5301 PC spectrofluorophotometer at 22 °C. The constants of drug–DNA binding equilibrium were calculated from the fluorescence spectra using Scatchard plots, that is, the ratio r/C1 as a function of r, where r is the concentration of the bound drug per base pair, and C1 is the concentration of free (unbound) DNA (bp).[18]

5.2.3. Antitumor potency of 1 and 3a

The antitumor potency of **1** and **3a** in vivo was studied on adult female hybrid mice (C57Bl/6 × DBA/2)F_1 (hereafter called B6D2F1) bearing T-cell lymphoma P388 or melanoma B16 transplants. Mice were bred at Kryukovo farm of the Russian Academy of Medical Sciences and hosted in the animal facility of Gause Institute of New Antibiotics. The experimental protocols were approved by the Committee on Animal Use and Care of Russian Academy of Medical Sciences. One million of tumor cells per animal were injected at day 0 (10 mice per group). Lymphoma P388 cells were inoculated ip, B16 melanoma cells were injected s.c. into the right flank. In the experiments with P388 tumor-bearing animals, the samples of **1** and **3a** were dissolved in 0.1% DMSO, diluted with saline and injected iv daily at days 3–7 or at days 1, 4, 7, 10 and 13 post tumor cell challenge. In the experiments with melanoma-bearing animals, **1** and **3a** diluted with saline were injected iv on days 1, 4, 7, 10, 13 post tumor cell inoculation (total 5 injections) or at days 1, 4, 7, 10, 13, 16, 19 and 22 (total 8 injections). In control cohorts the mice were left untreated. Daily monitoring of animals included the evaluation of general physical conditions, in particular, the changes of the body weight, behavioral and nutritional habits, and hair cover. Mice were sacrificed by day 28 post tumor challenge. The antitumor efficacy of **1** and **3a** was determined by the increase of lifespan (ILS) (P388) and inhibition of tumor growth (ITG) (B16). ILS (%) was calculated as $(D_t - D_c)/D_c \times 100$, where D_t is mean survival time (days) in drug-treated group and D_c is mean survival time (days) in control group. ITG (%) was calculated as $(M_c - M_t)/(M_c) \times 100$, where M_c and M_t denote mean calculated tumor weight (CTW) in the untreated (control) and drug-treated group, respectively. CTW (mg) was determined as $(a \times b \times c)/2$, where a, b, c are three largest orthogonal diameters (mm) of the tumor node.[19] Differences between CTW in drug-treated and untreated groups were evaluated using Student's t-test, with $p < 0.05$ considered as statistically significant.

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Supplementary data

Supplementary data (the ^{1}H NMR spectrum of compound **2** and HPLC for compounds **3** and **3a**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.055.

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